RESEARCHES INTO THE ETIOLOGY OF DENGUE

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ORIGINAL ARTICLES.

RESEARCHES INTO THE ETIOLOGY OF DENGUE.[1]

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The epidemic of dengue which prevailed throughout the State of Texas during the fall of 1885, was unusual in many respects, for example:

1st. In its universality.

2d. In the violence of its symptoms.

3d. In its manifest contagiousness.

4th. In the protracted convalescence of its subjects.

5th. Its hæmorrhagic tendency.

6th. In its numerous sequelæ.

From Galveston, located in the South-eastern portion of the State, it spread, in the course of a few months, to its Northern border. During this time almost every city, and many of the country districts within these limits, fell under the epidemic influence of this disease. In the city of Austin about 16,000 cases of dengue occurred during this time, out of a population of 22,000 inhabitants. I am informed that other cities and sections suffered as severely, *i.e.*, had as many cases in proportion to the population as did Austin.

Many cases where direct contagion was the cause of this disease came under the observation of the writer. He has no doubt that by this means it was carried and spread from one neighborhood to another, and from the city to the country by infected individuals. The violent type of the disease, the protracted convalescence of its victims, and its sequelæ, were unusually prominent in this epidemic. The *first*, by the suddenness of its invasion, often with an initial chill, its pyrexia, its excruciating pains, nausea, and the constancy of its exanthemata. The *second*, by the mental and physical prostration which follows its defervescence; this often continues for weeks or months, with anorexia and indisposition to perform any kind of labor.

The sequelæ most frequently observed were dysentery, entero-colitis, nephritis, bronchial and gastric catarrh, abscess, glandular enlargements and eruptions of the skin. It would seem from the peculiar clinical history of dengue, especially its contagiousness and its epidemic character, that it is a specific disease; that micro-organisms are the source of its infection, and these micro-organisms find in the blood a suitable environment for their growth and multiplication.

To determine the correctness of this theory, and if possible to obtain information with reference to the etiology and pathology of this malady, which is the scourge of our southern country, and especially as medical literature is absolutely silent upon these matters, the following experiments were conducted, with hopes that they would lead to a more rational therapy, or perhaps indicate some method of protecting individuals against dengue by means of inoculating them with the attenuated virus. I found, however, that I had neither the time nor means of carrying out the necessary investigations to determine the practicability of this hope, or of accomplishing these latter anticipations. A physician engaged in active practice has but little time, whatever his inclination may be, to indulge in experimental work. The demands of his patients and his creditors too frequently prevent his giving that time to original work or pure scientific labors which the interests of his profession demand.

During the six months occupied in making these investigations, one-half, and frequently two-thirds, of nights succeeding days of toil, were utilized in these labors; they possessed my interest; I managed to give them much of my time. The work actually performed during this time is embraced in the following statements:

1st. Blood, which was obtained from living subjects during the various stages of dengue, was microscopically examined, (a), directly, that is, without the addition of any chemical reagents; (b), after it had been subjected to the action of certain chemical reagents, viz: glacial acetic acid, with and without dilution; caustic potash in solution, both weak and strong; chloroform, and ether.

2d. This blood was carefully dried upon sterilized cover-glasses by passing these through the flame of a spirit lamp, and then subjected to the action of various staining reagents.

3d. Dengue blood obtained from living subjects was introduced, upon the point of a platinum wire, into test-tubes containing sterilized culture jelly prepared for this purpose.

These tubes were closed with plugs of sterilized cotton, then placed in an incubator, where the temperature was kept at 100° F. for the growth of such organisms as were contained in the blood.

4th. Blood was drawn directly from the veins of

[1] Read in the Section on Practical Medicine at the Thirty-Seventh Annual Meeting of the American Medical Association, St. Louis, May, 1886.

a living subject into a series of sterilized glass bulbs, which were united by a capillary-tube. This was performed in such a manner that it seems impossible for germs from the air, or by other accidental means, to have gained an entrance into these bulbs. These were also kept in the incubator, at a temperature of 100° F.

5th. The matters vomited and urine passed by dengue subjects were subjected to microscopic examinations.

It is the purpose of this paper to record the methods adopted in making these examinations, and the results obtained from them. Before entering into the technique of these methods, which are necessarily tiresome, I think it better to submit the following outline of the results obtained: In the blood examined directly, or after its treatment with the chemical reagents already referred to, stained or unstained, I invariably found, often in great numbers, in the cell elements as well as in the plasma, micrococci about $\frac{1}{20}$ to $\frac{1}{30}$ the diameter of the red cells, spherical in shape, and red or purplish in color; when these were seen in great numbers one layer superimposed upon another; frequently seen in the cultures, they appeared of a black or brownish color, but when seen singly or in thin layers in the blood or in the cultures, the red color is always distinct and characteristic.

During the development of this organism, at some period in its life-history, from causes which I do not understand, it becomes surrounded by a gelatinous envelope; this I have frequently observed in the blood and in the cultures alike. I always succeeded in growing in the culture-tubes, upon the surface of the jelly, micrococci, and no other form of bacteria, which in color, size and behavior, are identical with those seen in the dengue blood. The blood contained in the series of glass bulbs was examined, some after the lapse of six weeks, others at three months. In both instances I found that the blood contained a pure culture of micrococci which, in all respects, were the same as those which I had previously seen in fresh blood, and grown upon culture jelly, in culture tubes; these were apparently composed of micro-organisms held together by their gelatinous envelope, or capsules; at the end of the casts, where the micrococci were less firmly attached, or cemented together, their shape, size and color were found to correspond with those seen in the blood, or grown in the culture-tubes, or bulbs.

The microscopic examination of the blood for micro-organisms is attended with many difficulties. The first care is to obtain blood for examination that is not contaminated with bacteria from the air, or from other outside sources. In all examinations of the blood, or other portions of the body, for micro-organisms, every precaution against its accidental contamination, or the introduction of germs from outside sources, should be taken; without such precautions all investigations of this sort would be worthless and delusive.

When it is remembered that air and water contain large quantities of micro-organisms, that these adhere to the glass apparatus, metal instruments, fingers, and clothing of the operator, and that bacteria will quickly form in distilled water, and in staining fluids required in such examinations. the necessity of thoroughly washing and then sterilizing by heat and by chemicals, when this is practicable, all articles used, and by boiling and filtration, or other efficient means, becomes at once apparent.

The methods which I adopted to secure cleanliness and sterilization of all glass apparatus, instruments, and other agents used in these experiments, may not have been absolutely perfect in every instance. I made them as nearly so as the means at my command would allow. I am constrained to believe, from the uniformity of the results obtained, notwithstanding some minor defects in the detail of my technical methods, that, in the main, these were sufficiently exact to exclude serious errors.

The second difficulty which confronts him who examines blood for schizomycetes, is to dry the blood on the cover-glass without overheating, and thus disintegrating it.

The third difficulty is, to not mistake for micro-organisms certain elements and granules found in normal and pathological blood. These mistakes have occurred frequently, and wrecked many a beautiful hypothesis. That the reader may know and appreciate the difficulties to which I refer, I quote from the recent work of Dr. F. Hüppe:[1] "The examination of blood for bacteria offers very great difficulty, because in the normal blood within the vessels, and in the normal disintegration of the healthy blood, granular elements are present, or are formed, which under certain pathological conditions, in anæmic state and in fever, are increased in number, and can be easily mistaken for micrococci.

"They have already been often confounded with micrococci, and are almost daily mistaken for them, *e.g.*, the renowned syphilitic corpuscle, and the so-called organisms of the venom of serpents. Here belongs also much of what has been spoken of as the development of bacteria from nitrogen molecules, from microzymen, or from the anamorphosis of protoplasm. An exact study of these granules of the blood is, on this account, an indispensable desideratum in bacteria investigation.

"These granular forms further constituent parts of the cellular elements of the blood, and on this account again are of interest in ætiology, because there are parasites which are similar to the amœboid cells, *e.g.*, those monads found by Lewis in the blood of rats, by Koch in the blood of marmots. The elements of the blood, which directly or through their granules may be confounded with micro-organisms (with the exception of the red blood corpuscles), and the products of their disintegration), are divided according to Ehrlich into

"1st. Lymphoid elementis, (a), small lymph cells, (b), large lymph cells.

"2d. Myeloid cells (eosinophile).

"3d. Undetermined spleen and [or] marrow: (a), large mononuclear cells, (b), transitional forms, (c), polynuclear.

"The small lymphoid elements are somewhat

[1] "Methods of Bacteriological Investigation," by Dr. Ferdinand Hüppe. D. Appleton & Co., New York. 1886.

smaller than the red blood corpuscles, possess a very large nucleus, so that there is very little or no protoplasm to be seen.

"The large lymph elements are a further development of the first, and are only to be differentiated from them in this, that they possess, around the large nuclei, a distinct border of protoplasm.

"The myeloid elements are large round cells, with large oblong nuclei.

"The mononuclear cells are about three times the size of the red blood corpuscles, possess round or oval nuclei of large size, and a considerable mass of protoplasm. The mononuclear transitional forms are to be differentiated from these cells only in this, that the nuclei are no longer round or oval, but have become indented.

"The polynuclear elements are somewhat smaller, but still are always larger than the red blood corpuscles, and their nuclei show, as a further differentiation, a polynuclear form; these are the true white blood corpuscles.

"The granular elements, or granules, which are present in the cells, and which become free in the destruction of the same, are divided with respect to their reaction to aniline dyes.

"The α or eosinophile granule is coarsely spherical, strongly refracting, and can be stained in all the acid aniline dies. It is present in the myeloid elements, seldom in the normal blood, and its number is greatly increased in leucæmic processes.

"The β, or amphophile, is formed especially in the marrow, very often in the leucocytes in the blood of rabbits and guinea-pigs, and can be stained by acid and basic aniline dyes.

"The γ, or basophile plasma-cell granule, can be stained, like the bacteria, by the basic aniline dyes. These granules are coarse, slightly refracting, almost completely wanting in normal human blood, increased in leucæmic processes, and are present normally in the blood of the lower animals, especially the white rat.

"The δ, or basophile granule, is fine, and can be stained in basic aniline dyes, and forms a constituent part of the large mononuclear elements.

"The ε, or neutrophile granule, is very fine, and fills the polynuclear elements of the human blood quite thickly, is present sparsely in the transitional forms, and very seldom in the mononuclear elements. It can be stained by the neutral dyes. Without recourse to staining, these granules, as a whole, and also the products of the disintegration of the red blood corpuscles, may be confounded with micrococci.

"By systematic staining with aniline dyes the α, β and ε granules can be excluded. An error is then possible only with the γ and δ granules, because these are stained in the basic aniline dyes, the same as the bacteria. These last, on account of their fine grain, can, with comparative ease, be differentiated from micrococci, and have not, as yet, been confounded with them.

"The plasma-cell granules, on account of their medium size, come so near to the known forms of cocci that not only the individual free granules in the blood have been considered as cocci, but even the so-called plasma-cells in the tissues have been described as colonies of cocci. They can, on purely morphological grounds, be differentiated from them in this way, viz: That they do not all have the symmetrical appearance of the cocci, but present the greatest differences in the size of the granules.

"If it be desired to examine the blood for bacteria, a small drop is rapidly spread out in a thin layer, dried, fixed, and then passed three times through the flame, and then stained in the ordinary manner. In such preparations the bacteria are sufficiently stained, but not the granules."

Prolonged heating of cover-glass preparations, or sections containing these granules, is necessary to their successful staining. The method of doing this, which is recommended, is to subject the specimens, in a drying oven, to a temperature of 240° F. for an hour; they are then prepared for the staining process, and may be stained with their respective dyes, acid, basic or neutral, in the manner described.

As micrococci and other forms of schizomycetes do not require this prolonged heating in order to their successful staining with the aniline dyes, renders this difference in the behavior of the protoplasmic granules on the one hand, and micrococci on the other, an efficient means for their differentiation.

All the glass apparatus and instruments used in the following examinations of dengue blood were sterilized by the following methods: The cover-glass and microscope slides, which had never been previously used, were first washed in warm water with soap, then in clear water, then immersed in Seiler's cleansing fluid, prepared as follows:

Bichromate potash	2 oz.
Sulphuric acid	3 fld oz.
Water	25 fld oz.

When they were taken from this solution, they were again washed in clear water, and finally heated in the flame of a spirit lamp just before they were used. The metal instruments were washed in warm water and soap, clear water, and then in a 5 per cent. solution of carbolic acid; they were also heated in the flame of the lamp just before they were used. The platinum wire was fused to the end of a glass rod, this wire was sterilized always before using, by heating it to whiteness in the lamp. The arm or finger of the person from whom the blood was obtained was washed with warm water and soap, then in a 5 per cent. solution of carbolic acid.

The part was then punctured with a sterilized needle, and a small portion of the blood which appeared at the point of puncture was transferred by means of the sterilized platinum wire to the sterilized cover-glass, this was covered by another glass in order to obtain a thin layer of blood upon each of them; they were then separated, dried, and finally passed three times through the flame of the lamp, in order to thoroughly coagulate the albumen, and fix the blood elements.

Blood for examination was obtained from about forty typical cases of dengue, at various times and places, and during the various stages of the disease. The results which I obtained from examinations of these different specimens were entirely uniform.

Dengue blood dried upon the cover-glasses in the manner described was subjected to the action of certain chemical reagents, and aniline dyes, with the following results, viz.: some were treated with a 10 per cent. solution of caustic potash, others with glacial acetic acid, and others with ether and chloroform. None of these chemical agents had any destructive effect upon the micrococci which the blood contained—they could be seen as brilliantly and distinctly after treatment as before.

A peculiar effect was produced upon the microorganism by the glacial acetic acid. This agent stained these organisms brownish, and changed their shape from spheres to ovoids; these were seen in such numbers that the field of the microscope was covered by them. I think this change in shape was only apparent, and was caused by the action of the acid upon the capsules of the cocci. I am sustained in this opinion by the fact that the organisms seem larger after treatment with the acid; this was due to the invisible capsule being made visible by this agent. Inasmuch as there are no tissue elements in normal or pathological blood, as corpuscles, granules, cells or cell elements, which can resist the destructive action of each of these chemical reagents, whilst bacteria, on the other hand, can resist them, it would seem evident that if spherical bodies, uniform in size, shape, color and behavior, are found in the blood in certain pathological conditions, and these bodies can resist the destructive action of these chemicals, they must be micrococci, and cannot possibly be anything else. The remainder of these cover-glass preparations were subjected to the action of the various aniline dyes, in both weak and strong aqueous solutions, in aniline oil, water, and in alkaline solution.

The results which were obtained by these methods of staining were very instructive and important; for instance, it was shown:

First. That the dengue micrococci do not stain with the aniline dyes as readily as do other forms of bacteria.

Second. Methyl aniline blue in a weak solution of caustic potash furnishes a staining fluid for which the cocci of dengue manifest an especial affinity.

In order that the importance of this matter may be fully appreciated, I shall quote the following remarks from Friedländer:[1] "Furthermore, if among a certain number of elements which appear to be identical, some conduct themselves in a peculiar way toward a certain reagent, while others do not—if, for example, some are stained by a certain dye, while others remain colorless, we must necessarily conclude that there was a primary difference among the elements. Upon this principle are formed all the methods of preparation, and some of them very complicated, which are employed for the exhibition of the different histological elements. The principle in staining is, therefore, that certain elements or tissues, and also of cells, appropriate actively, or in large quantity, from the solution employed, a certain dye, and form with this a combination having an intense color, that is more or less permanent. . . . Hence in many instances staining assumes the importance of a chemical reaction, by means of which any particular structure that lies concealed among other bodies, can be brought easily into prominence.

"This 'elective' action of dyes is of extreme importance in pathological investigations. . . . The technique of dyeing is usually this: A section is transferred from distilled water to a dish filled with the staining solution, with which it is entirely covered; it remains in this for different lengths of time, varying from a few minutes to twenty-four hours, and is again immersed in distilled water, in order to wash away the portions of the dye that are adherent to its exterior.

"In many cases, however, the section which has been removed from the staining solution, and washed, is subjected to further manipulation; it is again decolorized, that is, partially. In this instance there has occurred at first a diffuse, even, but unnecessary amount of staining; but during the supplementary process of extraction, while certain elements give up their staining completely, others, that have a stronger affinity for the dye, retain it. This is called by Ehrlich the principle of maximum staining. In accordance with these principles of staining, the blood, which had been dried upon the sterilized cover-glasses, was submitted to the action of various aniline dyes, to find, if possible, some staining fluid with which isolated staining of the micrococci could be produced."

With this object in view, I tried successively Bismark brown, vesuvin, gentian, violet, methyl-violet, fuchsin, methyl-blue, aniline-green, picro-carmine, and eosin in watery solutions, and in solution with aniline waters. This result was obtained imperfectly with the solution of fuchsin in aniline water, and perfectly with methyl-blue in a solution of caustic potash.

With all the other dyes named, the results were negative, *e. g.*, all parts of the picture were stained alike—cells and organisms—and they were all decolorized with equal facility when washed in a 1 per cent. solution of acetic acid and then in absolute alcohol. With the methyl-blue potash solution, however, a very different result was obtained; this dye, in the solution referred to, manifested such an affinity, or elective action for the organisms of dengue, that these would retain the blue color after this had been extracted from the blood cells by the decolorizing agents named.

The manner of preparing this solution, and the method of staining with it, which were adopted, are as follows: Concentrated alcoholic solution of methyl blue, 30 c.cm.; solution of caustic potash, 1 to 10,000, 100 c.cm. In a dish filled with this fluid the cover-glasses were floated, with their blood sides downward. The dish was then covered to exclude dust, and the cover-glasses were kept in this condition from twelve to twenty-four hours. Better results are obtained by keeping the staining fluid during this time at the temperature of 100° F. The cover-glasses are then removed from the staining solution and washed in the 1 per cent. solution of acetic acid, then in absolute alcohol, until the color is entirely or sufficiently removed.

[1] The Use of the Microscope in Clinical and Pathological Examinations. By Dr. Carl Friedländer. D. Appleton & Co. 1880.

I think a better picture is obtained, and the relative position of the organisms to the cells shown, if the process of extraction is arrested before the cells are entirely decolorized; they should then be mounted in Canada balsam, and examined with a high power, and with a large diaphram or open condensor. The blood cells should show of a faint blue color, whilst the micrococci, which are to be seen in the blood cells and plasma, will be stained an intense blue. The inability of these organisms to hold the other aniline dyes, acid or basic, to which they were exposed, their uniform size, their presence in the blood cells, their ability to resist the destructive action of acids, alkalies, ether, etc., it would seem are sufficiently distinctive to differentiate them from protoplasmic granules, or the products of cell disintegration. An additional reason for regarding them as micro-organisms exists in the fact that they can and have been grown upon culture media, outside of the body.

In conducting these culture investigations, the same care to guard against error in results was practised as in the former. The test tubes, which had not previously been used for any purpose, were thoroughly washed with soap and water, then in clear water, dried, and plugged with absorbent cotton; they were then put into a furnace and exposed to a temperature of 400° F. for an hour. The culture medium which was used in all these investigations was that known as Miguel's lichen jelly; this was used in preference to gelatine, blood serum or other solid culture media for the following reasons:

It melts only between 55° and 60° C. (131° to 140° F.), which permits of the cultivation of such organisms as require for their development elevated temperatures. Ordinary nutritive gelatine melts before 30° C. (86° F.). Second. It remains without alteration or losing its power of solidifying when exposed to a temperature of 100° C. (230° F.) for rigerous sterilization. Gelatine, on the contrary, is reduced under such conditions to a turbid broth, which remains fluid on cooling. It is prepared by digesting Irish moss—*crispus chondrus*—in beef broth.

The broth is prepared as follows: Allow sixteen ounces of lean beef, as fresh as possible, two quarts of water, and sixteen grs. of table salt, to simmer for four hours in an uncovered vessel, then close it and boil for an hour, cool, and skim off the fat, neutralize carefully with carbonate of soda and filter.

In preparing the jelly, one ounce of Irish moss was digested in one quart of the broth, at a temperature short of boiling, the resulting decoction was passed through a colander to separate the swollen leaves, and then filtered through sterilized bolting cloth. It was then boiled for an hour, and whilst at this temperature the test-tubes were filled with this jelly, by means of a sterilized pipette, to about half their capacity; cotton stoppers were then introduced in the tubes, and these were subjected to a temperature of 212° F. in a Koch and Grafiky sterilizing cylinder for an hour each day for seven successive days.

After the last heating, the tubes were placed at an angle whilst the jelly cooled, in order that as large a surface as possible should be presented upon which the organisms could grow and multiply. Many test tubes were sterilized and filled with the jelly, and subsequently subjected daily to the temperature of boiling water in the manner above described. These were carried to the homes of those persons from whom dengue blood was obtained for inoculation purposes.

In obtaining this blood, every possible danger of introducing germs by accidental means was guarded against. The method of washing the arm and sterilizing the needle with which the puncture was made, has been described. A small wire of platinum, fused to the end of a glass rod, was used to convey a small quantity of the blood which appeared at the point of puncture into the test-tubes, where the point was brought in contact with the surface of the jelly. The only chance of introducing air germs occurred during the short time the cotton plug was removed to allow the passage of the platinum wire; the wire, of course, was heated to whiteness just before its use. Some twenty tubes of jelly were directly inoculated with the blood of dengue subjects; the blood used was obtained from different individuals, and always at their homes. These tubes were then put into an incubator, where the temperature was maintained constantly at 100° F., for the growth of the dengue organisms.

Without a single exception every tube which had been inoculated in the manner described showed, upon the surface of the jelly at the point of inoculation, a white spot elevated above the surface of the jelly; this gradually enlarged and could be seen, faintly outlined in the jelly, at the expiration of twenty-four hours. When this growth was examined under the microscope, with a high power, it invariably showed a pure culture of micrococci—which in color, size, shape and behavior, were identical with those seen in the blood of dengue subjects.

The uniformity of these results, in all the tubes inoculated, in growing these micrococci, and never other forms of bacteria, would certainly indicate that the matters of inoculation came from a common source, *e. g.*, the blood. If they had been accidentally inoculated from the air, or by the platinum wire, or through want of absolute sterilization of the tubes, many other forms of bacteria and fungi would have been found growing on the jelly, and these tubes, when opened, would have had the odor of putrefaction, which was not present in any of them. These cultures were continued from test-tube to test-tube for a period of six months. The method of perpetuating these pure cultures of the dengue micrococci, was to inoculate in the manner described, the sterilized jelly, in a fresh tube, from one which had previously been inoculated from the blood, then a third tube from the second, a fourth from the third, etc., through many generations of the organisms.

When it is considered that these culture investigations occupied a period of six months, and that during this time many transfers occurred from test-tubes containing micrococci to those which did not contain them; that each transfer removed by generations the organisms from those contained in preceding tubes; that frequent microscopic examinations of the differ-

ent series of tubes invariably revealed a pure culture, *e. g.*, without any other forms of bacteria; it would seem that the methods of sterilization employed had been successful in excluding alien germs, and that those found had a common origin from dengue blood.

The life history of these organisms was watched daily, and studied by means of Holman's life-slide. In the old cultures, those which were removed by many generations from the blood, the organisms were smaller, less deeply colored, and less frequently encapsulated, than those seen in the blood, or removed from it by a few generations. When examined in water without being stained, or in other fluid media which possess a low index of refraction, the micrococci are seen in active movement (Brown movement).

In those cultures which are removed by only a few generations from the blood, the cocci are seen surrounded by capsules. These are sometimes faintly colored pinkish, and can be distinguished from the cocci, which possess a deeper color.

Two methods of generation were observed: The first by fission; the organisms are seen to divide through their centres; each coccus will thus form two others. Frequently these newly formed organisms remain united together so as to assume the form of a rod or chaplet; this indicates that they belong to the class named streptococci. In other cases they unite to form swarms or zoogloæ. These latter become more compact, the distinctions of the cocci less, the color of the mass deeper, until they finally contract into corpuscular bodies, about the size of red blood cells; these often unite to form filaments, which frequently assume the shape of a network. This I have often seen when the culture was thinned with distilled water and allowed to dry upon a microscopic slide. This arrangement can be seen, whether the specimens are stained or not. The swarms and chaplets of the micrococci can be much better seen in the stained preparations. A histological stand, Abbé's illuminating apparatus and $\frac{1}{12}$ H. I. objective, manufactured by J. Grunow, of New York, were used in these researches.

The results obtained in the following and last series of investigations of dengue blood are to me very satisfactory and conclusive, inasmuch as the means adopted to exclude alien germs were, as far as I can see, absolutely perfect.

The apparatus used consisted of a series of glass bulbs united and blown upon a capillary glass tube—Liebig's potash bulbs. To one end of this apparatus was attached a new hypodermic needle, by means of a short piece of new rubber tubing. To the other end of the glass tube (which was packed with cotton) was attached an aspirator.

The following is the method used in sterilizing and using this apparatus, viz.: The series of bulbs were first chemically cleaned, one end of the tube was then packed with absorbent cotton, and the bulb heated in a furnace for an hour at 400° F. The rubber tubing, with the hypodermic needle attached, was treated with a 20 per cent. solution of carbolic acid, washed in clear water, and exposed to the temperature of escaping steam for an hour, in the sterilizing cylinder. It was then dried in the furnace, the free end of the tubing slipped over the free end of the glass tube, and the end to which the hypodermic needle was attached was encased with a sterilized test-tube, this was held in its place and the needle protected from air germs by means of absorbent cotton, that was packed firmly in the mouth of the test tube, and around the needle. The entire apparatus as thus arranged, tubing and needle attached, and covered with test-tube, was again put into the furnace and heated to 300° F. It was then removed from the furnace and carried to the home of the gentleman who kindly donated a sufficient amount of his blood for this investigation. He had a typical case of dengue. His arm was washed with soap and warm water, dried with a freshly ironed towel, and then a solution of carbolic acid, sufficiently strong to quickly whiten the skin, was applied to that part of the arm where the puncture was to be made. A ligature was then applied to the arm above the elbow. The test tube was removed from the hypodermic needle, and this was quickly passed into the large vein, at the bend of the arm. An aspirator was then attached to the free end of the glass tube—which was packed with sterilized cotton—and the bulbs partially filled with blood by means of aspiration. The glass tube, next to the rubber, was then closed and separated by the blow-pipe, before the needle was withdrawn from the arm.

These bulbs containing the dengue blood, which were effectually closed against the admission of all germs, and which contained no germs except those which entered with the blood from the veins of the donor, were then put into an incubator at 100° F. temperature, in order that a pure culture of the dengue micrococci might be grown in the bulbs, with their contained blood as the nutritive medium.

The first bulb was removed with the blow-pipe, and examined at the expiration of six weeks. The blood was examined, first, directly, without admixture; second, after treatment with glacial acetic acid, undiluted and in weak solutions; third, with 10 per cent. solution of caustic potash and in weak solutions of this alkali; and fourth, finally subjected to the various aniline dyes, before referred to.

In the direct examinations, a small drop of blood was placed upon the cover-glass, this was then inverted upon a slide, so as to obtain for examination a thin layer of blood; or the blood was examined in ⅔ per cent. salt solution or a solution of osmic acid 1 to 300. In those specimens examined directly, the field appeared to be covered with blood cells and dark pigment granules; these latter were seen in immense numbers.

When the blood was examined in the salt solution, or in the solution of osmic acid—this latter fixed the cells, and by giving them a faint brown color, rendered them more distinct—it was seen that what appeared as dark pigment granules in the direct examination, was now recognized as dengue micro-organisms, with all their distinctive features. The red cells in many cases were absolutely crowded with these, whilst in the plasma they were seen free, in swarms, zoöglœa, masses, and in corpuscular bodies, which sometimes united to form filaments, as in the

jelly culture. Cover-glass preparations of the blood, from this culture bulb, were subjected to the various aniline stains.

The indisposition to hold any of these dyes, except the methyl-blue potash, was as distinctly manifested as in the fresh dengue blood. The only difference in the appearance of the micrococci as observed in the fresh blood and that obtained from the culture bulbs is this: The organisms, when stained, appear larger in the latter than they did in the former; this, I think, is due to the fact that in the culture bulbs they are more frequently encapsulated. When both cocci and capsule are stained, the size is apparently larger.

The remaining bulbs were examined six weeks later. They all contained pure cultures of the dengue streptococcus, with disintegrated blood cells. These organisms were seen in zoöglœa, swarms, and in compact corpuscular masses; these were often united to form filaments. Normally shaped blood cells were found in only one of these last series of bulbs. I think their preservation in this bulb was due to a layer of coagulated serum which had formed over the surface of its contained blood. Many of the blood corpuscles in this bulb were more or less disintegrated, those which retained their shape were clouded by coagulation of the cell protoplasm, which concealed the organisms they contained to a greater or less extent. These, however, could be seen in great numbers, when the blood was examined in a weak alkaline or acid solution, which rendered the cells more transparent. None of the bulbs, when opened, had the least odor of putrefaction, and the only micro-organisms which they contained were those peculiar to dengue.

Pure cultures of these were found in each of the series of bulbs, and were seen in their various stages of development, *e. g.*, with and without capsules, in swarms, in zoöglœa masses and in corpuscular shaped bodies, frequently united to form variously shaped filaments. In these filaments the corpuscular masses, and often the cocci which formed them, could be seen faintly outlined.

This ends, at least for the present, these investigations. No one better knows than myself the incompleteness of this work, nor appreciates better the possibilities which might have resulted from its continued prosecution. The want of time and facilities for its proper execution prevented me from undertaking any additional labors in this matter. The investigations were undertaken without bias, and the conclusions arrived at are the result of careful, conscientious work. Whether these conclusions are true or false must be decided by the future.

I am greatly indebted to Dr. R. Munger, of San Antonio, Texas, for excellent micro-photographs of dengue blood, obtained from the first culture bulb. The zeal and patience displayed by him in this work are characteristic of the lover of science, while the excellent results obtained mark him as an expert in these matters. Many efforts were made by him to photograph the specimens colored with methyl-blue; success was attained only with those specimens which were stained after Sternberg's method for photographing micro-organisms, viz.:

A drop of sulphuric acid was placed upon the blood dried upon the cover-glass; this was allowed to remain for a minute, and then washed off with distilled water. The cover-glass was then floated, blood side downward, upon the following solution, in a watch crystal, viz.:

Iodine	grms.	3.
Iodide of potassium	"	5.
Distilled water	"	500.

After a few minutes' exposure the preparation will be found to present a deep orange color, which gives the desired contrast in a photographic negative. The micro-photographs which he obtained from specimens stained by this method displayed the blood cells, in which appeared many micrococci, while zoöglœa and swarms are seen in the blood plasma.

In conclusion, I desire to express the regrets I feel in not being able to complete these investigations. Zopf, Koch and others indicate among the questions to be answered in a thorough study of any bacteria, the following:

1. Shape, size, color and details of structure, *e. g.*, flagella, peculiar envelope, etc. Character and speed of movements.

2. Character of natural habitat. Artificial media best adapted to growth and reproduction. Stages of development passed through. Formation of zoöglœa spores, filaments, rods, cocci, "swarms." Conditions under which such formation occurs. Character of colonies formed in firm culture media.

3. Capability of producing fermentation, putrefaction. Character of decomposition products, volatile and other, formed in various nourishing media.

4. Behavior toward oxygen at normal and altered pressures. Behavior toward other gases.

5. Effects of various temperatures on movements, germination, etc.

6. Behavior in relation to light (phototonic properties). Behavior toward electricity.

7. Behavior toward antiseptics and poisons.

8. Are the forms under investigation found in a diseased organ or tissue. What is the effect of inoculating animals of different orders and species with pure cultures. If *virulent*, can the *virulence* be attenuated by exposure to air, to antiseptics, to heat, or by repeated "fractional" cultures. Under what conditions. Does the inoculation of attenuated germs have a *cumulative* effect if repeated at short intervals. Does one inoculation give immunity towards a second made with virulent microbes.

It will be seen that the character of investigation indicated by these questions could only be carried out by persons possessed of an abundance of time and money, and a willingness to devote both to such labors, or under the auspices and at the expense of the State or Federal Government. In view of the brilliant results already attained by such investigations, *i. e.*, the protection furnished, by inoculation with the attenuated microbes, against the following diseases, viz.: small-pox, hydrophobia, anthrax, yellow fever,(?) hog typhus and chicken cholera, it would seem a wise, humane and economical policy on the part of the State or Federal Government to encourage, by money appropriation, such investigations. Among the proba-

bilities which such investigations promise may be reckoned the prevention of many, perhaps all, the epidemic and contagious diseases to which man is heir.

Pasteur has astonished the world with his protective inoculations. His latest and most wonderful achievement is the immunity from hydrophobia which he claims is secured by inoculations with the attenuated virus.

Not less remarkable are the reputed discoveries of the yellow fever cryptococcus by Dr. Freire, of Rio Janeiro, and Dr. Carmona, of Mexico, and the immunity secured by inoculation with these organisms, as claimed by these gentlemen.

Other diseases and their specific microbes, notably diphtheria and phthisis, are under investigation by experienced bacteriologists, and in this day of marvels, we may expect soon to hear that these also are brought under the controlling influence of vaccination.

In the light of this experience, I feel secure in prophesying that within the next decade, dengue and many other contagious and infectious diseases will be as certainly prevented by inoculation as small-pox now is. It remains to be seen what contribution of knowledge will be furnished by America to this promising system of preventive medicine.